HISTOCHEMICAL AND BACTERIOLOGICAL STUDIES ON DIGESTION IN NINE SPECIES OF LEECHES (ANNELIDA: HIRUDINEA)

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Previous studies on digestion in the leeches (summarized by Herter, 1936; Harant and Grassé, 1959; Mann, 1962) have been restricted to a few genera, notably *Hirudo* and *Haemopis*. Such accounts as are available, however, indicate that the full complement of digestive enzymes normally present in the alimentary system is, in the leeches, much reduced. In *Hirudo*, for example, the careful experiments of Graetz and Autrum (1935) failed to demonstrate proteases in gut wall extract, and this supported earlier observations by Diwany (1925) that starved *Hirudo* could not digest milk, egg proteins or peptones injected aseptically into the gut. With *Haemopis* Autrum and Graetz (1934) and Graetz and Autrum (1935) showed that enzymes for initiating proteolysis (the endopeptidases of modern terminology) are absent, but that a number of those concerned in subsequent stages (exopeptidases) are to be found in extracts of the gut wall.

The alimentary system in leeches shows well developed regional differentiation in that a pharynx, oesophagus, crop, intestine and rectum are generally present, but, in contrast, there is little or no differentiation at the cellular level into glandular and absorptive components. This apparent lack of gland cells, apart from salivary glands, lends support to the biochemical evidence that digestive enzymes are reduced in number, although such absence of intercellular differentiation does not *ipso facto* eliminate the possibility of secretory activity in the gastrodermis.

The failure to demonstrate a full range of proteolytic enzymes led Graetz and Autrum (1935) to suggest that bacteria may be concerned in digestive processes, and this possibility was subsequently examined by Büsing (1951) and Büsing, Döll and Freytag (1953). A single species of bacterium, named by Büsing *"Pseudomonas hirudinis,"* was found consistently in the gut lumen of *Hirudo* and *in vitro* studies showed that the microorganism is capable of slowly digesting the blood which is the normal food of this leech. Inclusion of antibiotics in the food *in vivo* inhibited digestion. *"P. hirudinis"* was subsequently shown to be a species of *Aeromonas*, probably *A. liquefaciens* (Bullock, 1961).

Close association of bacteria with the alimentary system has been reported for a number of leeches in addition to *Hirudo* and they occur either in special oesophageal diverticula or intracellularly within the gastrodermis (Reichenow, 1922; Jaschke, 1933; Hotz, 1938). The organisms have not been shown to have a specific role in digestion, but their fairly widespread occurrence in the Hirudinea suggests that bacterial involvement in digestive processes may be a feature of this class of annelids.

Accordingly, a study has been made of digestive processes in nine species of leeches representative of the four major families and of varying predatory or para-

sitic habits. Histochemical and substrate-film methods have been used to ascertain whether there is, in fact, a reduction in the complement of digestive enzymes, and to localize and identify any enzymes still produced. Standard bacteriological methods have been used in parallel studies to detect any microorganisms present in the gut lumen, to characterize them as far as possible, and to assess their possible role in digestion from their behavior *in vitro*.

Materials and Methods

The following species of leeches, listed systematically and with an indication of their basic mode of life, have been examined:

Family Hirudidae

Hirudo medicinalis L. Fresh-water sanguivorous parasite, sucking blood principally from mammals but also from reptiles or amphibia.

Family Erpobdellidae

Erpobdella octoculata (L.). Fresh-water predator.

Family Glossiphoniidae

Glossiphonia complanata (L.). Fresh-water predator.
Helobdella stagnalis (L.). Fresh-water predator.
Theromyzon tessulatum (O. F. Müller). Fresh-water parasite on water fowl, sanguivorous.
Hemiclepsis marginata (O. F. Müller). Fresh-water parasite on fish and Amphibia, sanguivorous.

Family Piscicolidae

Piscicola geometra (L.). Fresh-water parasite on fish, sanguivorous.
Pontobdella muricata (L.). Marine parasite on fish, sanguivorous.
Platybdella anarrhicae (Diesing). Marine parasite on fish, sanguivorous.

Starved individuals and others fed at varying intervals before examination were relaxed in 10% magnesium chloride and then subjected to histological, histochemical or bacteriological studies.

Histological methods

Specimens fixed in Bouin, Susa or 10% neutral formalin were embedded in polyester wax (Steedman, 1957) and sections cut at 4 μ stained by haematoxylin and eosin, Mallory, periodic acid-Schiff, Feulgen, and the benzidine method for haemoglobin (Pickworth, 1934).

Histochemical methods

Enzyme activity was studied using frozen or 45° C. paraffin wax sections prepared after fixation at 4° C. in 10% formalin buffered to pH 7.0.

1. Endopeptidases. A positive reaction to the Hess and Pearse (1958) method for cathepsin C-like esterases has been used by Jennings (1962a; 1962b) and Rosenbaum and Ditzion (1963) as an indication of the presence of endopeptidases since it coincided with, and explained, observed progressive proteolysis in the gut of various invertebrates. In the absence of more specific techniques for endopeptidases this was adopted in the present study, using *o*-acetyl-5-bromoindoxyl acetate as substrate (Holt and Withers, 1952) and with preincubation of sections in 10^{-5} *M* E600 (diethyl-*p*-nitrophenyl phosphate) at 37° C. for 1 hour to inactivate B-type esterases, including lipases, which would otherwise give false positive reactions.

2. Exopeptidases. Aminopeptidase activity was visualized using L-leucyl-β-naphthylamide hydrochloride as substrate and Garnet GBC as simultaneous coupler (Burstone and Folk, 1956). When frozen sections were used they were first defatted in acetone to avoid diffusion of the fat-soluble reaction product.

3. Lipases. Possible lipolytic activity was investigated by the Tween 80 method (Gomori, 1952) and by the Holt and Withers (1952) method for esterases.

4. Carbohydrases. The ferric hydroxyquinoline method for β-glucuronidase as modified by Fishman, Goldman and Green (1964) was used to detect carbohydrases.

5. Alkaline phosphatase. Metal-salt and azo-dye methods (Gomori, 1952) were used to detect alkaline phosphatase, using sodium β-glycerophosphate and sodium α-naphthyl phosphate, respectively, as substrates and Fast red TR as coupler.

6. Acid phosphatase. This enzyme, often associated with intracellular digestion and lysosomal activities, was visualized in sections by the azo-dye method (Burstone, 1958) using naphthol AS–BI or AS–TR phosphates as substrates and Red violet LB as coupler.

Controls for histochemical methods included heat-inactivated sections, media lacking specific substrates, and the simultaneous processing of appropriate mammalian tissues.

Substrate-film methods

Thick frozen sections or bisected whole leeches, prepared after brief fixation in cold 10% neutral formalin, were tested for proteases by the silver halide-gelatine film method (Adams and Tuqan, 1961), for amylases by the starch-film method (Tremblay, 1963), and for lipases by laying upon tributyrin agar plates (Willis, 1960). Incubation at 22° C. was for 1 hour only, and heat-denatured material and drops of Seitz-filtered solutions of commercially available enzymes served as controls.

Bacteriological methods

1. Cultivation of the gut flora. The largest available individuals of each species were relaxed, pinned out and a hot soldering iron or scalpel used to sterilize the body surface over the gut region to be sampled. Ligatures were used to isolate regions of the gut, and on occasion the gut was exposed by dissection and its outer surface sterilized. Samples of gut contents were removed by forcing a fine Pasteur

pipette through the sterilized surface, and spread on nutrient agar. The agar plates were incubated at 25° C. for 24 hours together with controls inoculated from the sterilized surfaces, and only cultures whose corresponding controls showed no growth were retained for further study.

2. Hydrolytic capacities of the gut flora. The total hydrolytic effect of extracellular enzymes produced by microorganisms isolated from the gut was assessed qualitatively by culturing them, still as mass cultures not separated into pure strains, on appropriate media. Proteolytic activity was detected by blood agar, chocolate agar, skim milk agar, gelatine discs impregnated with charcoal, and Loeffler's serum. Lipases were detected by agar containing egg yolk, tributyrin or Tween 80 (Sierra, 1957) and amylases by starch agar. Blood agar served also for detection of haemolysins and egg yolk agar for lecithinases. Seitz-filtered solutions of commercial enzyme preparations acted as controls.

3. Characterization of components of the gut flora. Pure cultures were obtained by repeated subculturing, as necessary, from the original stocks and tested to gain some idea of the main types present. The tests used included growth on nutrient agar, blood agar and Loeffler's serum; nitrate reduction and citrate utilization; production of hydrogen sulfide, urease, catalase, oxidase or indol; the Voges Proskauer test; gelatine liquefaction; aesculin breakdown; growth in media containing glucose, lactose, sucrose, or starch; and sensitivity to penicillin and streptomycin. Details of these tests may be found in any standard bacteriology text.

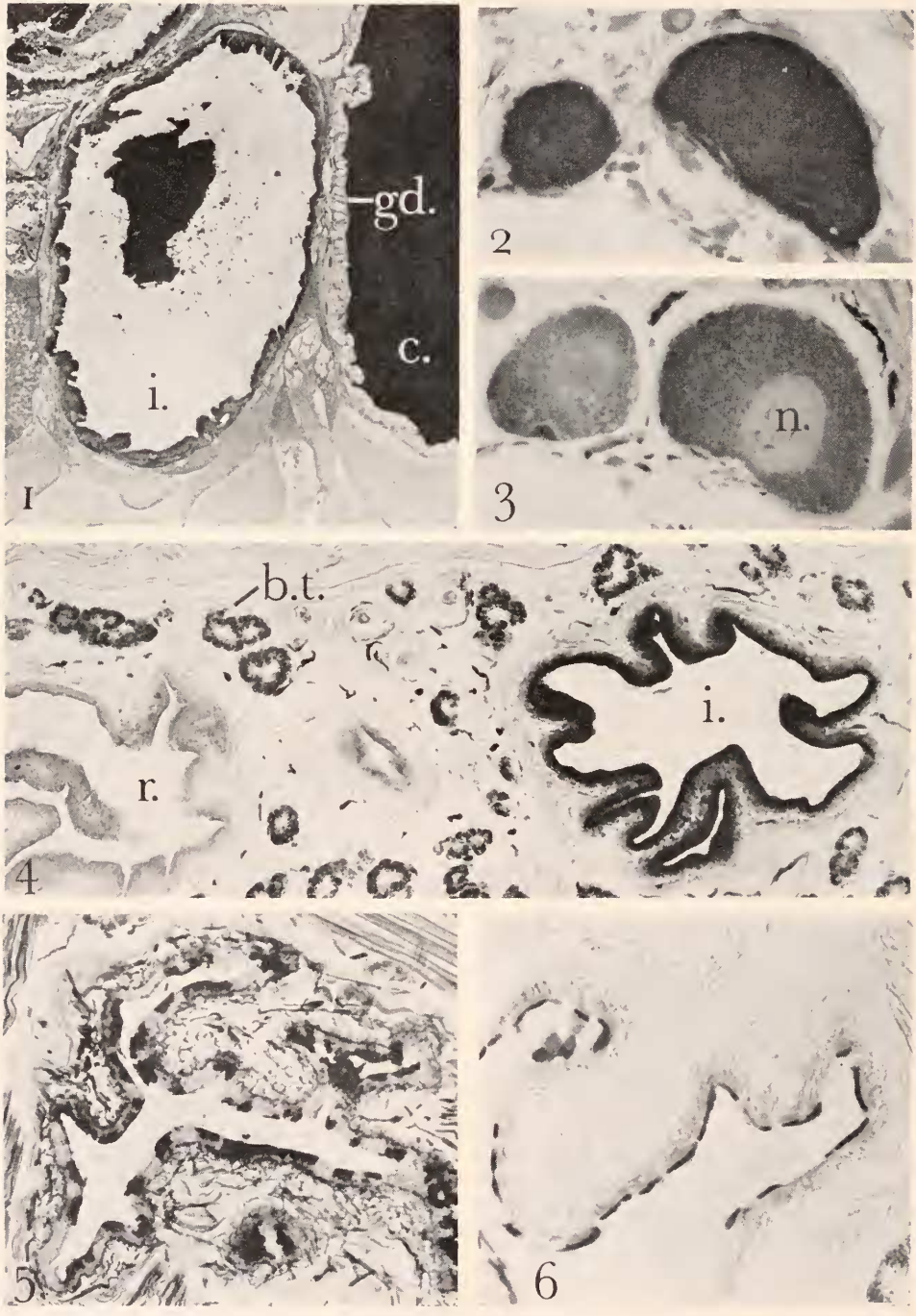
As controls *Pseudomonas fluorescens* was subjected to each series of tests, and other named strains were used in some individual tests. The tests selected included the majority of those used by Büsing (1951) to characterize *"P. hirudinis."*

Observations

General observations on feeding, gut structure and digestion

The six parasitic species feed on blood drawn from the respective hosts but the three predators feed on a variety of small invertebrates such as oligochaetes, other leeches, molluscs or insect larvae. *E. octoculata* swallows its prey intact but *H. stagnalis* and *G. complanata* use the proboscis to suck out fluids and soft tissues. The food is passed to the crop where it remains for periods varying from 1 to 2 days (*H. stagnalis*) to 6 months or more (*T. tessulatum*). Histological examination showed that there is no differentiation of the gastrodermis, in any of the species examined, into glandular and other components such as occurs in other annelids. The gastrodermis is relatively uniform in structure and there is no significant difference between that of the crop and intestine. A striated border, which stains strongly with the periodic acid-Schiff technique, is consistently present in both regions.

The course of digestion was traced in both parasitic and predatory species by following the fate of blood meals, the predators being induced to feed on clotted frog blood. Digestion in the gut lumen resulted in visible breakdown of erythrocytes, lysis of their nuclear membranes and release of nuclear constituents demonstrable in early stages by the Feulgen reaction but subsequently disappearing, and the degradation of haemoglobin into brown insoluble granules of haematin. In two species, *P. geometra* and *H. stagnalis,* there is extensive digestion in the crop



FIGURES 1–6.

but in all the others the significant amount of digestion occurs in the intestine, food being passed into this region a little at a time from the crop. Digestion in the intestine is predominantly extracellular but in *E. octoculata, G. complanata, T. tessulatum* and *H. marginata* the appearance of haem compounds in the gastrodermis (Fig. 1) suggests that there is also some intracellular digestion following absorption of materials from the gut lumen.

Occurrence and distribution of enzymes in the gut

1. Endopeptidases

Endopeptidases, as tested for by the Adams and Tuqan substrate-film method for proteases in general and the more specific histochemical method for E600-resistant esterases, could not be located in any part of the leech gut apart from in *P. geometra*. In this leech alone a large amount of esterase activity, some of it E600-resistant, occurs in the salivary glands (Figs. 2 and 3) and in the crop contents of recently fed individuals. None is produced by the crop gastrodermis, however, and that found amongst crop contents presumably originates in the salivary glands since leeches commonly inject saliva into host tissues during ingestion of the food. As noted earlier, digestion in *P. geometra* occurs largely in the crop and is much more rapid than in other sanguivorous species, erythrocytes which are prominent in the crop soon after feeding being completely digested within 10 days. It seems likely that salivary esterase is at least partly responsible for this breakdown.

In *P. anarrhicae* esterase activity was absent from the salivary glands but could be found on occasion in crop contents. Unlike the situation in *P. geometra*, however, activity was abolished by pre-treatment with E600. *P. anarrhicae* lives in the gill chamber of marine catfish and feeds principally on blood drawn from the gill capillaries, but some gill tissue and mucus are also ingested. Control sec-

FIGURE 1. *Theromyzon tessulatum*. Transverse section showing part of a diverticulum of the crop (c.) which projects posteriorly alongside the intestine (i.). The crop is filled with haemolyzed blood but its gastrodermis (gd.) shows no trace of absorbed haem compounds. In contrast there is little blood in the intestine and the intestinal gastrodermis is loaded with haem compounds absorbed from the lumen. Pickworth benzidine method for haemoglobin and haem compounds. Scale: 1 cm. = 100 μ.

FIGURE 2. *Piscicola geometra*. Transverse section through two salivary glands, both of which show an intense positive reaction for esterase. Hess and Pearse method without preincubation in E600. Scale: 1 cm. = 50 μ.

FIGURE 3. *Piscicola geometra*. Transverse section through the same salivary glands as in Figure 2, but showing some inhibition of esterase activity caused by preincubation of the section in E600. n., nucleus of gland cell. Hess and Pearse method with preincubation for 1 hour at 37° C. in 10^{-5} *M* E600. Scale: 1 cm. = 50 μ.

FIGURE 4. *Erpobdella octoculata*. Longitudinal section passing through the rectum (r.) and intestine (i.). The intestinal gastrodermis shows a strong positive reaction for aminopeptidase. The dark color of the botryoidal tissue (b.t.) is due to its endogenous pigmentation and does not represent a positive reaction. Burstone and Folk method. Scale: 1 cm. = 100 μ.

FIGURE 5. *Erpobdella octoculata*. Transverse section of the intestine showing the distribution of alkaline phosphatase in the gastrodermis. Gomori azo-dye method. Scale: 1 cm. = 50 μ.

FIGURE 6. *Erpobdella octoculata*. Longitudinal section showing the distribution of acid phosphatase in the striated border of the intestinal gastrodermis. Burstone azo-dye method. Scale: 1 cm. = 50 μ.

tions showed that gill tissue itself contains some non-specific and E600-sensitive esterase and this presumably persists for a time after ingestion by the leech. An identical occurrence of non-specific esterase of host origin has been recorded in the gut of the gill-dwelling monogenetic trematodes *Diclidophora merlangi* and *Octodactylus palmata* (Halton and Jennings, 1965).

In the majority of leeches prolonged incubation for 24 hours or more in the bromoindoxyl acetate medium produced scattered and very weak positive reactions in the intestinal gastrodermis. This was abolished by pre-treatment in E600, showing that the enzyme responsible is probably a B-type esterase and not a digestive endopeptidase.

2. Exopeptidases

In marked contrast to the negative results obtained for endopeptidases an extremely strong positive reaction to the Burstone and Folk method for aminopeptidase was given by the intestinal gastrodermis of all species except *H. medicinalis* and *P. muricata,* where somewhat weaker reactions occur. In the other seven species the intestinal gastrodermis shows an intense and continuous positive reaction (Fig. 4), and the most striking feature of this is the fact that it is always present irrespective either of the time elapsed since the previous meal or of the presence or absence of food in the lumen. It was found in every individual examined and was demonstrated with equal ease in both frozen and wax sections.

Material lying in the intestinal lumen on occasion showed a strong positive reaction, comparable to that of the gastrodermis. This extracellular activity clearly originated from the intestinal gastrodermis since neither the crop gastrodermis nor crop contents showed any trace at any time.

It is possible that the weak E600-sensitive esterase reaction given by the intestinal gastrodermis after prolonged incubation is caused by an aminopeptidase, as Smith and Hill (1960) state that pure leucine aminopeptidase shows slight esterase activity when tested biochemically against a number of substrates. On the other hand, Holt (1963) claims that this enzyme is resistant to E600 and if this is correct then the weak esterase activity of the leech intestine must be due to other, unidentified, causes.

3. Lipases

It was thought that lipases may have been partly responsible for the weak esterase activity found in the intestinal gastrodermis, since the reaction proved sensitive to E600, but Gomori's Tween 80 method failed to reveal any lipolytic activity anywhere in the gut, in any species. With the substrate-film method, frozen sections or bisected individuals of *G. complanata* and *P. geometra* caused slight clearing of tributyrin agar but the activity could not be localized to any one region of the gut.

4. Carbohydrases

No reaction was obtained to either substrate-film or histochemical methods for carbohydrases in any part of the gut, although the body musculature in most species gave positive results to tests for β-glucuronidase.

5. Alkaline phosphatase

Alkaline phosphatase, as demonstrated by the metal-salt and azo-dye methods, was found in the crop and intestinal gastrodermis in all species. Its distribution is somewhat patchy (Fig. 5) in that groups of cells show a reaction but these are interspersed with others showing little or no activity. Generally, the entire cytoplasm is involved in the cells showing activity, and there is no significant difference between cells of the crop or intestine.

The occurrence and distribution of alkaline phosphatase within the gastrodermis is quite independent of the nutritive state of the animal, precisely as in the case of aminopeptidase.

Slight reactions were obtained in the salivary glands of *H. medicinalis, T. tessulatum* and *P. geometra* and in the last two species the enzyme occurs also in the integument over the entire body except for the adhesive surfaces of the suckers.

6. Acid phosphatase

The distribution of acid phosphatase, as traced by the Burstone azo-dye method, followed closely that of alkaline phosphatase except that it could not be demonstrated in the crop of *H. marginata*. The enzyme occurs generally in the striated border, but occasionally extends well into the distal half of the cell. The distribution throughout the gastrodermis is discontinuous (Fig. 6), as in the case of alkaline phosphatase, but, again, it is consistently present irrespective of the presence or absence of food.

In *P. geometra* the salivary glands, which produce the salivary esterase, show at all times a strong reaction for acid phosphatase, and in *T. tessulatum* occasional cells in the salivary glands of young individuals show a similar response.

Bacteriological results

1. Hydrolytic capacities of the gut flora

Eight leech species were investigated in this part of the study, *H. stagnalis* being omitted since insufficient specimens were available. Samples from crop and intestine were obtained separately whenever possible but in some instances it was impossible to state categorically that samples came from one region alone, due to the size of the animal or movement of gut contents prior to ligaturing and sterilization. Cultures obtained from samples withdrawn under these circumstances are designated in the summaries of results as from the "gut," as opposed to others known to originate specifically in the crop or intestine.

The number of attempts to establish mass cultures from the different regions of the gut, and the number of successes, are summarized in Table I. Various factors probably account for the fairly high proportion of failures but a significant one, no doubt, was sterilization of gut contents during the essential sterilization of tissues prior to removal of samples.

The results of testing the mass cultures by each of five methods for proteases, three for lipases and one for amylases are summarized in Tables II and III. Table IV summarizes results of tests for haemolysins which produced β-haemolysis type color changes on blood agar, and tests for lecithinases on egg yolk agar.

TABLE I

Summary of attempts to establish mass cultures of bacteria from the leech gut
(a., no. of attempts; s., no. of successes)

	"gut"		crop		intestine		total	
	a.	s.	a.	s.	a.	s.	a.	s.
H. medicinalis	12	6	6	6	2	2	20	14
E. octoculata	16	12	9	3	11	7	36	22
G. complanata	9	8	7	7	6	5	22	20
T. tessulatum	16	8	27	6	2	2	45	16
H. marginata	2	2	5	2	1	1	8	5
P. geometra	12	4	16	7	1	0	29	11
P. muricata	—	—	2	2	—	—	2	2
P. anarrhicae	18	4	—	—	—	—	18	4

TABLE II

Summary of tests for proteases on mass cultures of gut bacteria

When only a proportion of the cultures established were tested the number used is shown after the number of positive results, otherwise the total number established were tested.
b.a., blood agar; c.a., chocolate agar; m.a., milk agar; c.g. charcoal gelatine; L.s. Loeffler's serum.

	Cultures established	Nos. effecting hydrolysis on					Overall %
		b.a.	c.a.	m.a.	c.g.	L.s.	
H. medicinalis							
"gut"	6	6	6	6	6	6	100%
crop	6	6	6	6	6	6	(70:70)
intestine	2	2	2	2	2	2	
E. octoculata							
"gut"	12	10:10	2:2	8:8	10:10	8:8	100%
crop	3	3	3	3	3	3	(84:84)
intestine	7	7	5:5	6:6	6:6	7	
G. complanata							
"gut"	8	6:6	6	7	5:7	8	87%
crop	7	7	4:6	5	5:6	7	(83:95)
intestine	5	5	5	5	4	4	
T. tessulatum							
"gut"	8	4	2:4	5	7	4:4	57%
crop	6	4	1	2:2	2	5	(38:66)
intestine	2	2	0	0	0		
H. marginata							
"gut"	2	2	1	2	2	1:1	95%
crop	2	2	2	2	2	2	(23:24)
intestine	1	1	1	1	1	1	
P. geometra							
"gut"	4	4	4	4	4	4	84%
crop	7	7	3	7	3	3:3	(43:51)
P. muricola							
crop	2	2	1	2	2	—	87% (7:8)
P. anarrhicae							31%
"gut"	4	2	1	0	0:2	2:2	(5:16)

If the organisms tested play a dominant part in the digestive processes of their hosts, then a success rate approaching 100% would be expected, *i.e.*, it is reasonable to expect each replicate mass culture such as the six established from crop contents of *H. medicinalis* to show a hydrolytic effect on each substrate. Controls

TABLE III

Summary of tests for lipases and amylases on mass cultures of gut bacteria
e.y.a., egg yolk agar; t.a., tributyrin agar; Tw. a., Tween 80 agar; st.a., starch agar.

	Cultures established	Nos. effecting hydrolysis on				Overall % for	
		e.y.a.	t.a.	Tw.a.	st.a.	lipases	amylases
H. medicinalis							
"gut"	6	6	6	6	6		
crop	6	6	6	6	6	100%	100%
intestine	2	2	2	2	2	(42:42)	(14:14)
E. octoculata							
"gut"	12	12	12	12	12		
crop	3	3	3	2	3	97%	100%
intestine	7	6	7	7	7	(64:66)	(22:22)
G. complanata							
"gut"	8	5:6	4:5	5:7	8		
crop	7	4	6	4:6	7	79%	100%
intestine	5	4	5	5	5	(42:53)	(20:20)
T. tessulatum							
"gut"	8	1	3	1	0		
crop	6	2:5	6	1:5	1	39%	6%
intestine	2	0	2	2	0	(18:46)	(1:16)
H. marginata							
"gut"	2	2	2	2	0		
crop	2	1	2	2	1	86%	40%
intestine	1	1	1	0	1	(13:15)	(2:5)
P. geometra							
"gut"	4	2:2	2:3	2:2	4	96%	36%
crop	7	6:6	6:6	7	0	(25:26)	(4:11)
P. muricata							
crop	2	1	2	2	1	83% (5:6)	50% (1:2)
P. anarrhicae							
"gut"	4	2	3	0	0	41% (5:12)	0 (0:4)

of Seitz-filtered bacteria-free solutions of commercial pepsin, pancreatin and lipase did, in fact, give this expected 100% success rate.

The results show that in two species, *H. medicinalis* and *E. octoculata*, there is substantial evidence for the presence of a gut flora capable of taking a leading part in the leech's digestive physiology. Cultures from *H. medicinalis* gave scores of the expected 100% in tests for proteases, lipases and amylases, and those from

E. octoculata were the same apart from a score of 97% (64 positives out of 66 tests) for lipases. The other species, however, provided cultures showing varying degrees of discrepancy. Material from *G. complanata,* for example, gave relatively high scores (87% for proteases, 79% for lipases and 100% for amylases), but with *T. tessulatum* the score was only 57%, 30% and 6%, respectively, and with *P. anarrhicae* it was 31%, 41% and nil.

TABLE IV

Summary of tests for haemolysins and lecithinases on mass cultures of gut bacteria

	Cultures established	Numbers positive for		Overall % for	
		haemolysins	lecithinases	haemolysins	lecithinases
H. medicinalis					
"gut"	6	6	1		
crop	6	6	6	100%	64%
intestine	2	2	2	(14:14)	(9:14)
E. octoculata					
"gut"	12	10:10	9		
crop	3	3	0	100%	54%
intestine	7	5:5	3	(18:18)	(12:22)
G. complanata					
"gut"	8	5:5	0:6		
crop	7	3:5	2:5	80%	25%
intestine	5	4	2	(12:15)	(4:16)
T. tessulatum					
"gut"	8	3	0		
crop	6	1:3	1:5	30%	6%
intestine	2	0	0	(4:13)	(1:5)
H. marginata					
"gut"	2	0	0		
crop	2	0	2	20%	60%
intestine	1	1	1	(1:5)	(3:5)
P. geometra					
"gut"	4	4	1	36%	54%
crop	7	0	5	(4:11)	(6:11)
P. muricata					
crop	2	0	0	0	0
				(0:2)	(0:2)
P. anarrhicae					
"gut"	4	2	1	50%	25%
				(2:4)	(1:4)

The complete conformity with the expected 100% in the *H. medicinalis* cultures and in the majority of those from *E. octoculata* suggests that the nonconformity of figures from the other six species is significant. Thus it would appear that while populations of bacteria are present which possess considerable hydrolytic abilities it seems unlikely that the microorganisms participate in digestive activities to the same extent as in *H. medicinalis* or *E. octoculata.* Nevertheless, their

hydrolytic abilities are such that the possibility of their participation to some lesser extent cannot be denied.

The conclusion that the gut bacteria cannot be the sole agent in the digestive processes of most leeches is supported by the fact that their hydrolytic abilities vary considerably both between and within host species. In *T. tessulatum,* for example, cultures established from different individuals do not always affect blood agar, chocolate agar, milk agar or charcoal gelatine; thus there are no grounds for asserting that in *T. tessulatum* the gut flora at all times possesses components which together can attack a wide range of proteinaceous substrates. Similarly, in *P. geometra* only blood agar, milk agar and Loeffler's serum were consistently attacked by cultures from all animals tested, and the frequency with which other substrates were attacked varied considerably between individuals.

The activities designated in Table IV as "haemolysins" and "lecithinases" have not been included in the above observations since the enzymes responsible represent specialized classes of proteases and lipases, respectively. Both types could conceivably be of importance in the digestive physiology of sanguivorous leeches, with lecithinases concerned in breakdown of erythrocyte walls and haemolysins being perhaps especially important in degradation of haemoglobin. Thus if the gut flora participated extensively in digestive processes it could be expected that all cultures would show these activities, at least in sanguivores. *H. medicinalis,* however, was the only sanguivore yielding cultures showing 100% positives for haemolysins. The same value was obtained for lecithinases in cultures known to have originated from either the crop or intestine, but of those from the "gut" only 1 out of 6 were positive. Inclusion of these "gut" results reduces the total incidence of positives for these enzymes in *H. medicinalis* to 64%.

E. octoculata, a predator and non-sanguivore, resembles *H. medicinalis* in respect of the high haemolysin and lecithinase activities of its gut flora, but in the other leeches these activities are more limited and even in sanguivores the score was consistently low. In *T. tessulatum* and *P. geometra,* for example, the score for haemolysins was only 30% and 36%, respectively.

2. Characterization of components from the gut flora

A full bacteriological investigation of the flora of the leech gut was beyond the scope of the present study, but a number of standard tests were applied to pure cultures of Gram-negative rods isolated from "gut" samples of *H. medicinalis, E. octoculata, T. tessulatum, H. marginata* and *P. geometra.* A synopsis of the tests and results obtained is given in Table V. Interpretation of the results is based on data given by Breed, Murray and Smith (1957); Hugh and Leifson (1953); Klinge (1960); Park (1962); Rhodes (1959); and Shewan, Hobbs and Hodgkiss (1960).

A number of the pure strains broke down Hugh and Leifson's glucose medium oxidatively, *i.e.* under strictly aerobic conditions. This is a characteristic of most species of *Pseudomonas* and strains ER1 and ER2 (*E. octoculata*), HM1 and HM2 (*H. marginata*), and PI1 and PI2 (*P. geometra*) are identified as such, their other properties as summarized agreeing with this. Strains TH1 and TH2 (*T. tessulatum*) also attacked Hugh and Leifson's medium oxidatively, but are not identified as pseudomonads as they failed to utilize citrate. They are tenta-

tively identified as species of *Xanthomonas* since although they lack the characteristic pigmentation their properties agree best with *Xanthomonas* data.

The majority of the remaining strains broke down Hugh and Leifson's medium by fermentation, with production of gas, indicating *Aeromonas* sp. They included HR1 and HR2 from *H. medicinalis* and this identification agrees with data given by Bullock (1961) for the identification of *"Pseudomonas hirudinis"* (Büsing,

TABLE V

Synopsis of tests on pure cultures from the leech gut
HR *H. medicinalis;* ER *E. octoculata;* TH *T. tessulatum;* HM *H. marginata;* PI *P. geometra.*

	HR		ER						TH		HM		PI	
	1	2	1	2	3	4	5	6	1	2	1	2	1	2
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mobility	+	+	+	+	+	+	+	−	+	+	+	+	−	−
Polar flagella	1	1	3	3	1	1	1	−	1	1	1	1	3	3
Reaction in media containing:														
glucose (H. & L.)	F	F	Ox.	Ox.	F	F	F	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.
lactose	AG	AG	−	−	−	−	−	−	A	A	−	−	−	−
sucrose	AG	AG	−	−	A	A	A	−	A	A	−	−	−	−
Starch hydrolysis	+	+	−	−	+	+	−	−	−	−	−	−	−	−
Aesculin breakdown	+	+	−	−	−	+	+	−	+	−	−	−	−	−
Citrate utilization	+	+	+	+	+	+	+	+	−	−	+	+	+	+
Nitrate reduction	+	+	−	+	+	+	+	+G	+	−	+	−	−	−
Formation of:														
indol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S	+	+	−	−	+	+	+	−	+	+	−	−	−	−
urease	−	−	+	+	+	+	−	−	−	−	−	−	−	−
oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pigment	−	−	Gr.	Gr.	−	−	−	−	−	−	−	−	−	−
Gelatine liquifactn.	+	+	+	−	+	+	+	−	+	+	+	+	+	+
Voges Proskauer	+	+	−	−	+	−	+	−	−	−	−	−	−	−
β haemolysis	+	+	−	+	+	+	+	−	+	+	+	+	−	−
Fluorescence in U.V.	−	−	+	+	−	−	−	−	−	−	+	+	+	+
Sensitivity to:														
penicillin	−	−	−	−	−	−	−	−	−	−	−	−	−	−
streptomycin	+	+	+	+	+	+	+	−	−	+	+	+	+	+

−, negative; +, positive; F, fermentation with gas production; Ox., oxidative breakdown; A, acid production; G, gas production; Gr., green pigment produced.

1951) as an *Aeromonas,* probably *A. liquefaciens.* Strains ER3, ER4 and ER5 (*E. octoculata*) were the others effecting fermentation and identified on the basis of this and other properties as *Aeromonas* sp.

The remaining strain from *E. octoculata,* ER6, was an encapsulated form producing abundant slime, and is identified as a species of *Klebsiella.*

The principal fact emerging from these tests, then, is that the predator *E. octoculata* harbors more species of microorganisms than the sanguivorous leeches examined, all of which yielded only one species. Further, the types obtained from the sanguivores varied with the particular host species, *H. medicinalis* yielding

only *Acromonas* sp., *H. marginata* and *P. geometra* giving species of *Pseudomonas*, and *T. tessulatum* species of *Xanthomonas*. In contrast, *E. octoculata* yielded species of both *Pseudomonas* and *Acromonas*, as well as of *Klebsiella*.

All these types of bacteria are commonly found in the fresh-water habitat of the various leeches.

Discussion

Previous reports that the gastrodermis in leeches lacks morphological differentiation into secretory and absorptive components have been substantiated in the present study, where histological and histochemical examinations have failed to reveal any glandular structures. Further, in contrast to most other animals, the leeches do not appear to produce endopeptidases in any part of the alimentary system, apart from in the isolated case of *P. geometra* where an E600-resistant esterase forms part of the salivary secretion. It is impossible, however, to state categorically that endopeptidases are absent, since Vanha-Pertula, Hopsu, Sonninen and Glenner (1965) have shown that the endopeptidase cathepsin C when freed from all impurities fails to hydrolyze significant amounts of the bromoindoxyl acetate which was used as a substrate in the search for these enzymes in the leech. It is unlikely, though, that the leech gastrodermis would produce a protease of this type alone, and the techniques used here have certainly revealed endopeptidases or endopeptidase-like enzymes in other invertebrate groups (Jennings, 1962a, 1962b; Rosenbaum and Ditzion, 1963).

In marked contrast to the apparent absence of endopeptidases a strong positive reaction to the Burstone and Folk method for aminopeptidase is universally obtained in the intestinal gastrodermis. This reaction is, in fact, indicative of the presence of several exopeptidases, including leucine aminopeptidase for which the method was originally believed to be specific, as it has been shown that the L-leucyl-β-naphthylamide substrate can be split by a range of amino-peptidases and by at least one carboxypeptidase (Sylvén and Bois, 1962, 1963; Sylvén and Bois-Svensson, 1964). Such occurrence of exopeptidases in the gastrodermis at all times and hence independently of the nutritive state of the leech is quite different from the situation in other invertebrates such as flatworms and nemerteans where an aminopeptidase reaction can be demonstrated only at specific times after feeding (Rosenbaum and Rolón, 1960; Jennings, 1962a, 1962b). In these animals the reaction is entirely intracellular but in the leeches it is also found extracellularly, and this may account for the fact that the enzymes responsible can always be demonstrated in the intestinal gastrodermis. There is strong evidence that the exopeptidases are concerned in intracellular digestion, as well as extracellular, since haem compounds were found in the gastrodermis, apparently undergoing digestion, in a number of cases.

The occurrence of acid and alkaline phosphatases, like that of the exopeptidases, is unrelated to the nutritive state of the leech. These enzymes are probably concerned with absorption of material from the gut lumen, as they occur consistently in the striated border of both crop and intestinal cells. In the latter the phosphatases are probably concerned in uptake of materials for intracellular digestion, but their role in the crop gastrodermis is less clear. In sanguivores water, salts and soluble metabolites such as glucose may be absorbed from the blood meal as it lies

in the crop, and in *P. geometra* there is probably absorption of products of the digestion effected in the crop by salivary esterase. The crop is primarily a storage organ but in most animal groups possessing such a structure a few members have extended its use to include digestion effected by enzymes either swallowed as part of the saliva or regurgitated from the intestine (Jennings, 1965), and it is interesting to find that the leeches are no exceptions to this general rule.

The evidence that exopeptidases are the only endogenous proteases in the species examined agrees with similar findings for *Haemopis* by Autrum and Graetz (1934) and Graetz and Autrum (1935), and supports the idea that this may be characteristic of the Hirudinea as a whole. This, however, poses the problem of the mechanism of proteolysis which obviously cannot follow the same course as in other animals where endopeptidases and exopeptidases act in sequence. The hypothesis of bacterial involvement has been tested and in two species there is convincing evidence that microorganisms may be extensively involved in digestive processes, including proteolysis. In the other species the gut flora possesses more limited hydrolytic abilities and presumably, therefore, participates to a lesser extent in digestion. It is conceivable that the exopeptidases of the leech continue and complete proteolysis initiated by the gut flora, but it is perhaps more likely that the principal role of these enzymes is independent of the gut symbionts. Thus digestion of protein may well be effected by a series of exopeptidases, of various group and bond specificities, which between them progressively remove terminal amino residues from the protein chains. This type of activity has, in fact, been demonstrated *in vitro* by Hill and Smith (1958, 1959, 1960) who showed that pure leucine aminopeptidase can remove, stepwise, 109 of the 185 amino acid residues of papain and hydrolyze completely the polypeptide glucagon. Comparable activity in animal digestive processes would be slow and inefficient without initial intervention of endopeptidases to provide a greater number of terminal units for exopeptidase attack, but, in favor of this hypothesis, digestion in leeches *is* a slow and much extended process.

Support for this interpretation of the basis of leech digestive physiology can be obtained, paradoxically, from *H. medicinalis* where the gut flora seems to be capable of extensive participation in digestion. In this leech aminopeptidase activity in the gastrodermis is relatively weak, indicating perhaps that more reliance is placed on the gut flora than on endogenous enzymes. In *E. octoculata,* the other species in which bacteria could be extensively concerned in proteolysis, there is the more typical strong reaction for endogenous aminopeptidase so that in this species perhaps both types of mechanism operate. In *E. octoculata* a more varied gut flora is present, but this is probably related to the predaceous feeding habits which allow a wide selection of microorganisms to enter the gut with each meal and, no doubt, some of these become established in the new habitat.

Since no endogenous amylases or lipases have been found in the leech gut it is possible that the bacterial flora is concerned in digestion of these substances. The mass culture tests showed only restricted amylolytic and lipolytic activities, but carbohydrates and fats do not preponderate in the diets of either predatory or parasitic leeches. It seems obvious that bacteria are important in leech nutrition, in view of their fairly general occurrence (Reichenow, 1922; Jaschke, 1933; Hotz, 1938), and in addition to their suggested participation in digestion they may also

contribute vitamins, probably of the B group, to the host economy since these will be scarce in the diet of sanguivorous leeches, if not in that of predators. A comparable situation exists in sanguivorous arthropods (summarized by Wigglesworth, 1965), where symbionts contribute significant quantities of vitamins to their hosts.

If this interpretation of the present evidence is correct, then it would appear that the leeches during their evolution have for some reason lost the capacity to produce endopeptidases, and probably also lipases and amylases. In compensation for lack of endopeptidases production of exopeptidases has been emphasized and these enzymes, aided by a symbiotic gut flora, have become responsible for the entire sequence of proteolysis. As a further adaptation the leeches have developed a tolerance of the consequent slowing down and extension of digestion, and adaption of sanguivorous habits with considerable lengths of time elapsing between meals has fitted in well with this type of digestive physiology.

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Summary

1. Histological, histochemical and bacteriological methods have been used to study digestion and the nature and source of digestive enzymes in nine species of leech.

2. The gastrodermis is not differentiated morphologically into secretory and absorptive structures and there is little difference in the structure of the crop or intestine.

3. Endopeptidases, which initiate proteolysis in most animals, lipases and amylases do not appear to be produced by the leech digestive system.

4. The possibility that the gut flora is concerned in digestion in compensation for the lack of endogenous enzymes, has been investigated using mass cultures of the microorganisms normally present in crop and intestine. In two species the combined hydrolytic capacities of the gut flora are considered sufficient for it to play an important part in digestion of proteins, fats and carbohydrates; and in the other species there is evidence that it can participate to a lesser but still significant extent.

5. Exopeptidases, as typified by the presence of aminopeptidases, are produced in the intestinal gastrodermis and can be consistently demonstrated irrespective of the nutritive state of the animal. There is evidence that they act both intra- and extracellularly.

6. It is suggested that the exopeptidases play a part different from their normal one in animal digestive physiology, in that they act in the absence of endopeptidases by slowly degrading protein chains by progressive removal of terminal units. This proteolysis supplements any effected by the gut flora.

7. Acid and alkaline phosphatases are consistently present in the gastrodermis of the leech and are believed to be concerned with absorption of material from the gut lumen.

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